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**MIGRATION PATTERNS OF DENDRITIC CELLS IN THE  
MOUSE**

**Homing to T Cell-dependent Areas of Spleen, and Binding within  
Marginal Zone**

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We have demonstrated (1) that splenic dendritic cells (DC) migrate from peripheral blood to some host lymphoid and nonlymphoid tissues and particularly the spleen, entry to which depends on the presence of T lymphocytes. DC do not have access to lymph nodes from the blood, but migrate into them via the afferent lymphatics independent of T cells. Here we have used fluorescent techniques to define the localization of adoptively transferred DC within the spleen. We also describe an assay to study the interaction of DC with frozen sections of tissues. These techniques have enabled us to clarify the relationship between isolated lymphoid DC and the interdigitating cells (IDC) of T cell-dependent areas, and how T cells may regulate the entry of DC to lymphoid tissues.

**Materials and Methods**

The preparation of DC and T lymphocytes, and labeling with Indium-111 [<sup>111</sup>In]-tropolone, were as described (1); C57/BL10 (H-2<sup>b</sup>) mice were used routinely. Unfractionated resident peritoneal cells (2) were a source of macrophages (Mφ).

*Hoescht 33342 (H33342) Labeling.* Cells were resuspended at  $\sim 5\text{--}10 \times 10^6/\text{ml}$  in RPMI 1640 (RPMI) containing 2–5% FCS, and incubated with a blue-fluorescing, DNA-binding fluorochrome, Hoescht 33342 (H33342, 6  $\mu\text{g}/\text{ml}$ ; Sigma Chemical Co., Poole, United Kingdom) for 15 min at 37°C (3).  $3\text{--}10 \times 10^5$  T cells were injected in serum-free RPMI into (ether-) anesthetized recipients, intravenously via the penile vein or subcutaneously into one footpad.

*Localization of H33342-labeled DC within Specific Areas of Lymphoid Tissues.* Spleens or popliteal nodes were dissected from recipients of H33342-labeled cells. Frozen sections (10  $\mu\text{m}$ ) were prepared immediately, or after storage of the tissues at  $-30^\circ\text{C}$ , and fixed in acetone at 22°C for 10 min. They were incubated with mAbs specific for Ia<sup>b</sup> (B21-2; reference 4) or Lyt-1 plus Lyt-2 (references given in 1), followed by FITC-rabbit anti-rat Ig (Dako Ltd., High Wycombe, United Kingdom) plus 2% mouse serum. Sections were viewed under an ultraviolet microscope and photographed using ASA 400 film and 5-s and 30-s double exposures for blue and green fluorescence, respectively.

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**Frozen Section Assays.** H33342-labeled cells were resuspended in RPMI containing 1% FCS at room temperature; in some experiments 10  $\mu\text{g}/\text{ml}$  DNase (Sigma Chemical Co.) was also added.  $1-3 \times 10^5$  cells in 7  $\mu\text{l}$  were placed on freshly cut, unfixed, frozen sections on four-spot slides (C. A. Hendley Ltd., Essex, United Kingdom). They were covered with tube caps and incubated at 4°C on ice, or 37°C in a humidified incubator for 30 min, 3  $\mu\text{l}$  serum-free RPMI being added after 15 min to compensate for evaporation. Excess medium was then carefully aspirated, and the cells were fixed in 120  $\mu\text{l}/\text{section}$  1% formalin in PBS without calcium or magnesium for 10 min at room temperature. Sections were gently rinsed in changes of RPMI until only firmly attached cells remained; they were viewed by UV microscopy and photographed. Similar results were obtained after 45-min incubations, and if cells were not fixed in formalin although the morphology was less clear.

### Results and Discussion

**DC Migrate from the Blood and Home to T Cell-dependent Areas of Spleen.** Sorted DC were labeled with the fluorochrome, H33342, to allow their visualization in relation to particular areas of tissue sections that were defined by mAbs and FITC-conjugated anti-Ig; anti-Lyt-1 plus anti-Lyt-2 was used to label T cells, and anti-Ia for IDC and B cells. 3 h after intravenous injection, 75% of DC were located in the red pulp, and only 10% were within T-dependent areas (Fig. 1, A and C). In marked contrast, by 24 h at least 65% of DC were within T areas, while 10% remained outside (Fig. 1, B and D); DC were in areas containing IDC and were clearly excluded from the B cell areas (Fig. 1 E).

These results were confirmed in independent experiments by photographic analysis. Some 15–25% of cells could not be localized precisely, being either too close to the edge of the main T areas, or associated with small, isolated groups of T cells, particularly at 24 h. The same findings were made (not shown) when T and B areas were identified by antibodies to Thy-1 plus L3T4 (CD4), and RA3-2C2/1 plus RA3-3A1/6.1 or anti-Ig (1).

The simplest explanation is that DC actively migrate from the blood into the red pulp of spleen and thence to T-dependent areas to become IDC. H33342-labeled T cells, for comparison, were localized in T-dependent areas by 3 h, and persisted at 24 h (not shown). DC thus have characteristic migration patterns distinct from those of T cells, B cells that migrate to B areas, and large granular lymphocytes and unfractionated bone marrow cells that remain in the red pulp.

In preliminary experiments, a similar analysis of the cells entering the popliteal nodes from the footpad after subcutaneous transfer was carried out (results not shown). DC were predominantly outside T areas at 3 h and tended to be localized towards the subcapsular region, but many were within T areas by 24 h. But the situation appears to be more complex than in spleen and is being investigated. T cells were already associated with the T cell-dependent areas of node by 3 h, and persisted at 24 h (not shown).

**Control Experiments.** Control studies (data not shown) established that DC function was not affected by the labeling procedures; these controls are also relevant to the accompanying paper where we followed the traffic of  $^{111}\text{In}$ -labeled DC (1). First, the distribution of cells double labeled with  $^{111}\text{In}$  and H33342 was similar to those labeled with  $^{111}\text{In}$  alone. Second, H33342-labeled cells were fully capable of stimulating the allogeneic mixed leukocyte reaction. Third, C57/BL10 hearts, transplanted as heterotopic, vascularized allografts, were rejected at 17 d (median survival time, MST) by fully allogeneic DBA/2

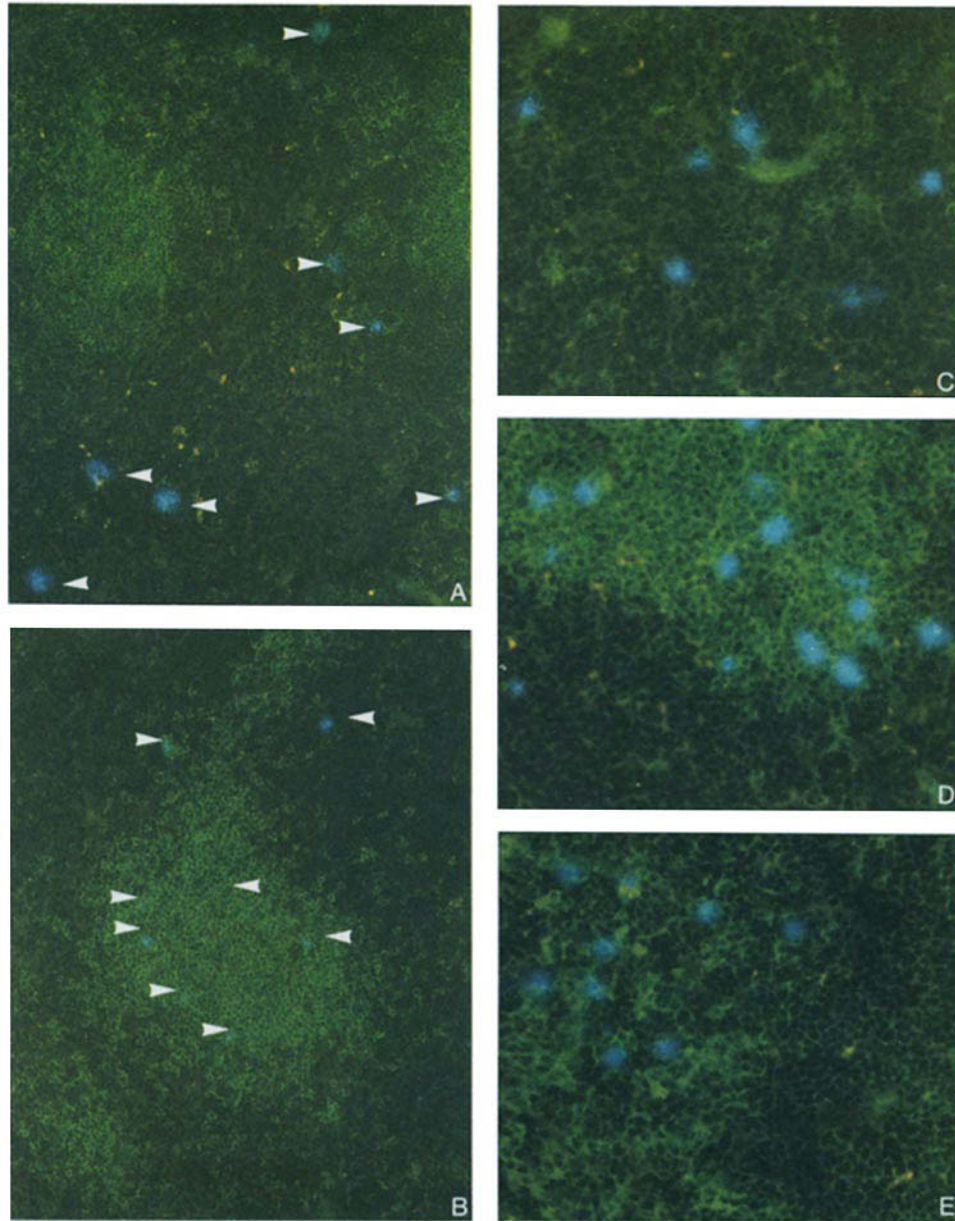


FIGURE 1. Homing of dendritic cells to T cell-dependent areas of the spleen. Sorted DC were double labeled with  $^{111}\text{In}$  and (blue) H33342, and injected intravenously into syngeneic recipients. At 3 h (A and C) and 24 h (B, D, and E) spleens were removed. Frozen sections were stained with rat mAbs specific for T cells (A–D) or Ia (E), followed by (green) FITC-anti-rat Ig. Low (A and B) or higher power (C–E) fields were viewed by ultraviolet microscopy and photographed. The radioactivity in spleen and other tissues was measured to confirm that the quantitative migration patterns were similar to those reported previously (1) for DC labeled with  $^{111}\text{In}$  alone (data not shown).

recipients (5). Pretreatment of these with  $1.5 \times 10^5$  C57BL/10 DC, 3 d before transplantation, led to accelerated rejection at MST 4 d; the same dose of double-labeled DC induced rejection at MST 5 d ( $n = 5$ ). Additionally, there was no labeling in the spleen when cell-free H33342 was injected intravenously. Our results here and elsewhere (1) therefore reflect the migration of functionally active DC.

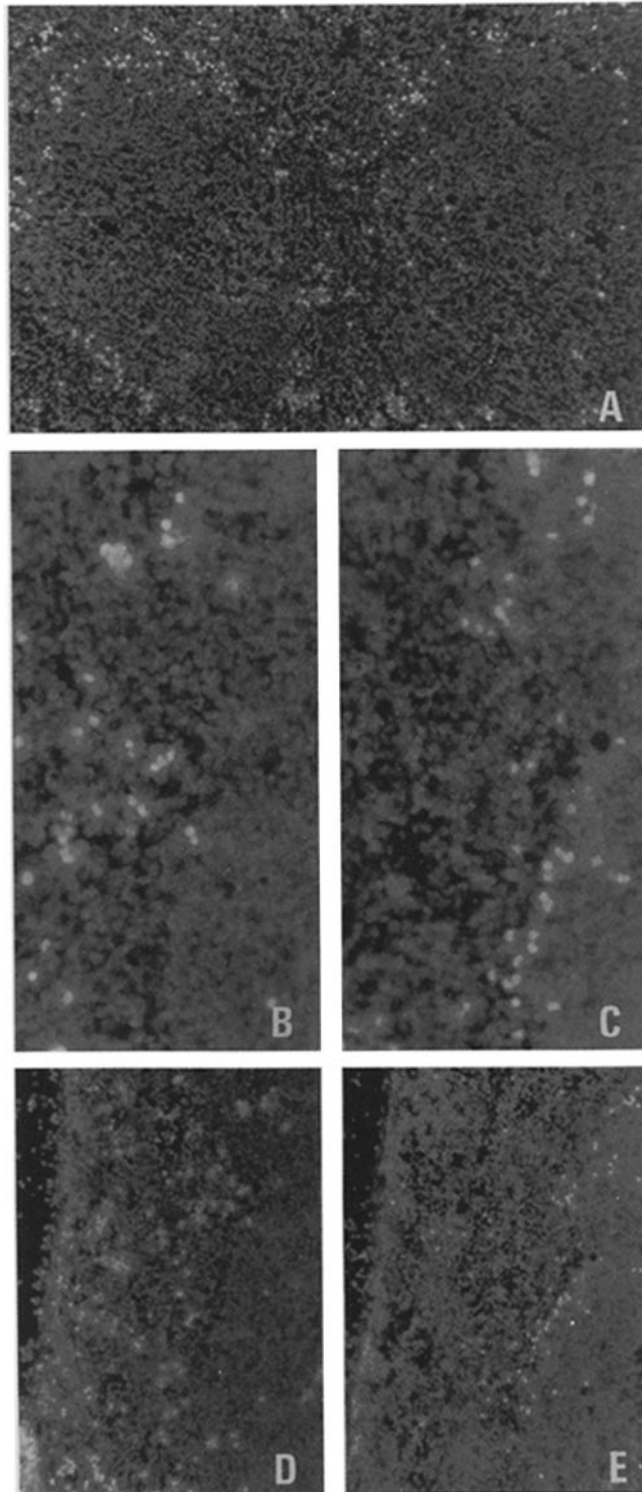
*DC Can Adhere to the Marginal Zone of Spleen.* Entry of DC from blood to spleen depends on the presence of T cells (1). We initially hypothesized that this could be due to clustering of the DC with T cells, similar to that studied in vitro, but the fact that DC are first localized in the red pulp (Fig. 1, A and C) argues against this notion. To determine whether DC can interact with specific regions of spleen we developed a frozen section assay (see Materials and Methods) based on that used to examine binding of T lymphocytes to high endothelial venules (HEV) (6).

DC (EA<sup>-</sup>) that were incubated at 37°C on frozen sections of spleen mostly adhered to the marginal zone and were excluded from the white pulp. Obvious "crescents" of attached cells were evident around these areas in 10 of 12 experiments after 30–45 min (Fig. 2A); occasionally a few DC also attached to the red pulp. No binding whatsoever occurred at 4°C (not shown). In contrast to DC, T cells either did not bind to spleen sections or a small number adhered nonspecifically (not shown), and M $\phi$  adhered mainly to red pulp at 37°C and to a lesser extent at 4°C (compare Fig. 2, B and C, and D and E).

The attachment of DC and T cells to frozen sections of lymph nodes was also examined (not shown). A small number of DC bound but were not obviously localized to specific areas, whereas T cells adhered to discrete regions in the cortex at 4°C but not at 37°C, presumably HEV (6). Although we cannot exclude the possibility that this assay might be relatively insensitive, there seems to be a correlation between the ability of DC to bind to lymphoid tissues in vitro and whether they have access to these sites (spleen, node) from the blood in situ (1).

We currently assume that DC adhere to endothelium rather than to marginal zone M $\phi$ , and would propose that DC can express endothelium-specific "homing receptors" akin to those of T cells. Presumably, in vivo, DC that leave the blood circulation first attach to the endothelium within the splenic marginal zone before crossing to the red pulp; here they may undergo some development before eventually migrating into the white pulp to become IDC. The striking T dependency of DC entry to the spleen (1) suggests that T cells or their products might modify the endothelium to allow recruitment of DC from the circulation. Conceivably this could occur at nonlymphoid sites where T cells have accumulated, for example in response to a chronic inflammatory response.

What is the relationship between isolated lymphoid DC and IDC within T areas? Although for long thought equivalent, it is notable that the only detectable staining by an anti-mouse DC antibody, 33D1, was within the marginal zone of spleen (7) [although two other antibodies labeling IDC, veiled cells, and Langerhans' cells also labeled isolated DC (8)]. Conceivably, isolated lymphoid DC are not directly equivalent to IDC, but represent a migratory form that is transiently localized within the marginal zone and/or the red pulp of spleen and which may also be present in the blood (see also discussion to reference 1).



**FIGURE 2.** Binding of dendritic cells to marginal zone of spleen. EA<sup>-</sup> DC (A, C, and E) or Mφ (B and D) were labeled with H33342 and incubated on frozen sections of spleen at 37°C as described in Materials and Methods, and photographed through ×10 (A, D, and E) or ×25 (B and C) objectives. Serial sections are shown in B–D, with the white pulp towards the right; B and D are higher power views of part of C and E, respectively. Note the brightly labeled DC that have attached in the marginal zone between the more densely packed white pulp and the more diffuse red pulp (A, C, and E). In contrast, Mφ have attached mainly to the red pulp (B and D). The section itself is weakly labeled because some fluorochrome leaches from the cells.

### Summary

Using quantitative techniques we have shown elsewhere that dendritic cells (DC) migrate from blood into the spleen, under the control of T cells. Here we traced the localization of DC within the spleen and sought to explain the means by which they entered. DC were labeled with a fluorochrome, Hoescht 33342, and injected intravenously. Spleens were removed 3 or 24 h later and DC were visualized within particular areas that were defined by mAbs and FITC anti-Igs. At 3 h most DC were in the red pulp, whereas by 24 h the majority had homed to T-dependent areas of the white pulp and may have become interdigitating cells. Lymphoid DC, isolated from spleen and perhaps normally present in blood, may thus be a migratory stage distinct from the relatively fixed interdigitating cells.

We also developed a frozen section assay to investigate the interaction of DC with various lymphoid elements. When DC were incubated on sections of spleen, at 37°C but not at 4°C they attached specifically within the marginal zone and did not bind to T areas; in contrast, macrophages attached only to red pulp and T cells did not bind specifically. However, DC did not bind to sections of mesenteric lymph node, whereas T cells localized in particular regions at 4°C but not at 37°C, probably the high endothelial venules. DC may thus express "homing receptors," similar to those of T cells, for certain endothelia. We propose that T cells can modify the vascular endothelium in certain areas to allow egress of DC from the bloodstream.

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